Preliminary Aspects of Genetic Management for Pacific Threadfin *Polydactylus sexfilis* Stock Enhancement Research in Hawaii

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Abstract

Preliminary aspects of genetic management for Pacific threadfin stock enhancement research at the Oceanic Institute (OI) have been focused on genetic stock identification and broodstock management. To investigate genetic structure in threadfin populations potentially impacted by stock enhancement, wild specimens from 4 locations in Hawaii (n = 41) and from 3 locations in Oahu (n = 32) were assayed by sequencing 1045 base pairs of the mitochondrial DNA (mtDNA) control region. Due to the large number of nucleotide sites assayed, haplotype diversity was high (99.3%); a total of 61 unique haplotypes were observed from the 73 individuals assayed. However, nucleotide diversity was low (0.64%). No phylogeographic structure was evident in clustered haplotypes. Genetic variance was partitioned predominantly among individuals within populations (98%); approximately 1% of the genetic variance occurred between the threadfin from the islands of Oahu and Hawaii. Haplotype distributions did not differ significantly among these two locations. These data, which are preliminary, are suggestive of high gene flow on a regional basis. The female effective population size, estimated using a Maximum Likelihood Metropolis-Hastings sampling method, ranged approximately 200,000-400,000. The sampled population appears to have undergone a large, historical expansion. Taken together, data are consistent with an evolutionarily recent colonization of the species in the Hawaiian Islands. Preliminary studies for broodstock management are focusing on levels of relatedness among female broodstock and female contributions to OI progeny groups. Using mtDNA sequencing, maternity surveys were performed for cultured progeny groups which contained both normal individuals and individuals exhibiting a particular morpho-anatomical deformity. The condition appeared to be manifested ubiquitously and randomly among progeny of the contributing females. Data provided no evidence that inbreeding or maternal effects are causative factors. If controlled by a single autosomal (dominant or recessive) gene, our preliminary results suggest that the condition has very low penetrance/expressivity in the wild threadfin population.

Introduction

The Pacific threadfin, *Polydactylus sexfilis* (Valenciennes *in* Cuvier and Valenciennes, 1831), is widely distributed throughout the Indo-Pacific region. Because of recent morphological studies, the taxonomy of *Polydactylus* is currently in flux (*see* Mishra and Krishnan, 1993; Fricke, 1999; Motomura *et al.*, 2000; Motomura *et al.*, 2001). Fricke (1999) suggested that *P. sexfilis and P. sextarius* should be synonymized. Motomura *et al.* (2001) disagreed, offering a revised morphological description of *P. sexfilis* based on comparisons of type material and new specimens collected range-wide to specimens (including relevant type material) from the putative congeneric species *P. kuru*, *P. nigripinnus*, *P. plebeius*, and *P. sextarius*. Interestingly, in Motomura's *et al.*, (2001) study, type material for *P. kuru* was indistinguishable from specimens of *P. sexfilis* and thus the authors regarded *P. kuru* to be a junior synonym of the latter. The western margin of the Pacific Plate represents a biogeographic boundary for the majority of *Polydactylus* species (Springer, 1982). Of approximately 20 threadfin species in the Indo-Pacific, the Pacific threadfin is one of two or three species that have colonized the interior portion of the plate. It characteristically occurs near oceanic islands and is considered to be less dependent upon large river systems than its regional congeners.

In the Hawaiian Islands, Pacific threadfin is a popular sport fish that also supports localized commercial fisheries. Unfortunately, annual catches of threadfin in some areas have declined dramatically over the last few decades and have not recovered despite implementation of stringent fishery regulations. Beginning in 1993, the Oceanic Institute undertook an experimental program to determine if cultured Pacific threadfin can be used to replenish depleted Hawaiian fisheries (reviewed in Ziemann, 2002, this issue). Between 1993-1998, cultured threadfin were tagged and released along the windward coast of Oahu in order to study factors critical to survival and recruitment (e.g., the effects of various stocking densities, size-at-release, and release habitat; Friedlander and Ziemann, in press; Ziemann and Friedlander, in press). Early results were promising – cultured threadfin comprised approximately 10% threadfin in Oahu's recreational catch in the years following stocking. As part of the Oceanic Institute's effort to implement a responsible program of stock enhancement, current research involves the genetic study of native and cultured Pacific threadfin.

Among the cultured threadfin recaptured in coastal waters were several large, fully-mature females. Where there exists an opportunity for reproductive exchange between cultured and wild organisms, there also exists the potential for genetic impact. Tringali and Leber (1999) outlined three types of genetic hazards that should be considered when cultured and wild stocks are mixed: Type I -- among-stock (introgressive) transfer of deleterious genes; Type II -- pre-release genetic modification of cultured organisms (including domestication, purging of diversity, and inbreeding); and Type III -- genetic swamping of recipient stocks from overwhelming contributions of cultured organisms. To manage these genetic risks, a baseline genetic survey of the potentially impacted wild population is necessary. This survey should include the following components: 1) quantification of levels of genetic diversity in the population, 2) geographic/spatial partitioning of genetic variation, 3) quantification of temporal genetic variance (drift) and estimation of effective population sizes, and 4) characterization of the population's demographic history.

The mitochondrial genome offers a ready source of genetic characters for many of the above-listed analyses. Mitochondrial DNA is relatively easy to obtain and assay (Palumbi, 1996); there is no need for expensive and time-consuming marker development and testing.

MtDNA is haploid, typically maternally inherited, and not subject to recombination. MtDNA generally evolves in a "clocklike" fashion in percoids, although relative rates of mtDNA sequence evolution may vary among congeneric species (e.g., Tringali *et al.*, 1999). Because rates of nucleotide substitution in mitochondrial genes of vertebrates are 5-10 times higher than in most nuclear genes (Wilson *et al.*, 1985; Billington and Hebert, 1991), resolution in population-level mtDNA studies is generally good. In particular, examination of nucleotide variation in the non-coding "control region" of mtDNA has proven to be a powerful tool for studying genetic structure in marine species (e.g., Stepien, 1995; Graves, 1998; Reeb, 2000). Moreover, this marker has been used to delineate interbreeding wild fish stocks prior to enhancement (e.g., Seyoum *et al.*, 2000; Garber, 2001), to compare levels of genetic diversity between hatchery and wild stocks (e.g., Sekino *et al.*, 2002, *this issue*), to determine effective sizes for hatchery cohorts (M. Tringali, unpublished data), to identify a significant maternal component to red drum hatchling survivorship during "common-garden" artificial rearing (M. Tringali, unpublished data), and to track hatchery organisms after release (e.g., Fujii, 2002, this issue).

Here, we report the preliminary results of a survey of mtDNA control region sequence for wild Pacific threadfin from two regions within the Hawaiian Island chain. Sequence data were also obtained for members of a threadfin progeny group produced at the Oceanic Institute to investigate if there was a relationship between maternity and a recurrent anatomical defect in hatchlings. Data collection and laboratory analyses are ongoing. The resultant information will be useful in the development of a risk-adverse plan of genetic management for the Pacific threadfin stock enhancement program.

Material and Methods

Sample Collection and Laboratory Analyses

Locations of capture and sample sizes for wild specimens are given in Fig. 1. In addition, a total of 49 threadfin, reared from captive broodstock at OI, were assayed. These represented a subsample of progeny that had been pooled at hatching from two OI spawning groups. Twenty-four of the cultured specimens exhibited a particular developmental defect – i.e., a missing or deformed opercle. The remaining 25 specimens appeared to be developmentally normal.

Total genomic DNA was obtained from each specimen by using an organic extraction procedure based on that of Taggart *et al.* (1992). The resulting DNA pellets were resuspended in 50 µL of TE buffer (10 mM Tris, 1 mM EDTA; pH 8.0) and quantified using fluorescence spectrophotometry, as described by Gallagher (1994). DNA concentrations were then adjusted to 100 ng/µL using 1 mM Tris and stored at -20 C. Species-specific primers designated *ThreadPro* (5'-CCTACTGCTTCAAAGAAGAG-3') and *ThreadPhe* (5'- TTGTGCTCACAGGGGTTGTC-3') were designed (K. Stuck and W. Grater, unpublished) and used to amplify the entire mtDNA control region. PCR amplifications were conducted in replicate 50 µL reactions containing 200 ng template DNA, 1.5 mM $MgCl_2$, 200 µM deoxynucleotide triphosphates (dNTP), 0.3 µM of each primer, and 3.0 units of *Taq* DNA polymerase with 10X PCR buffer (Amersham Life Science).

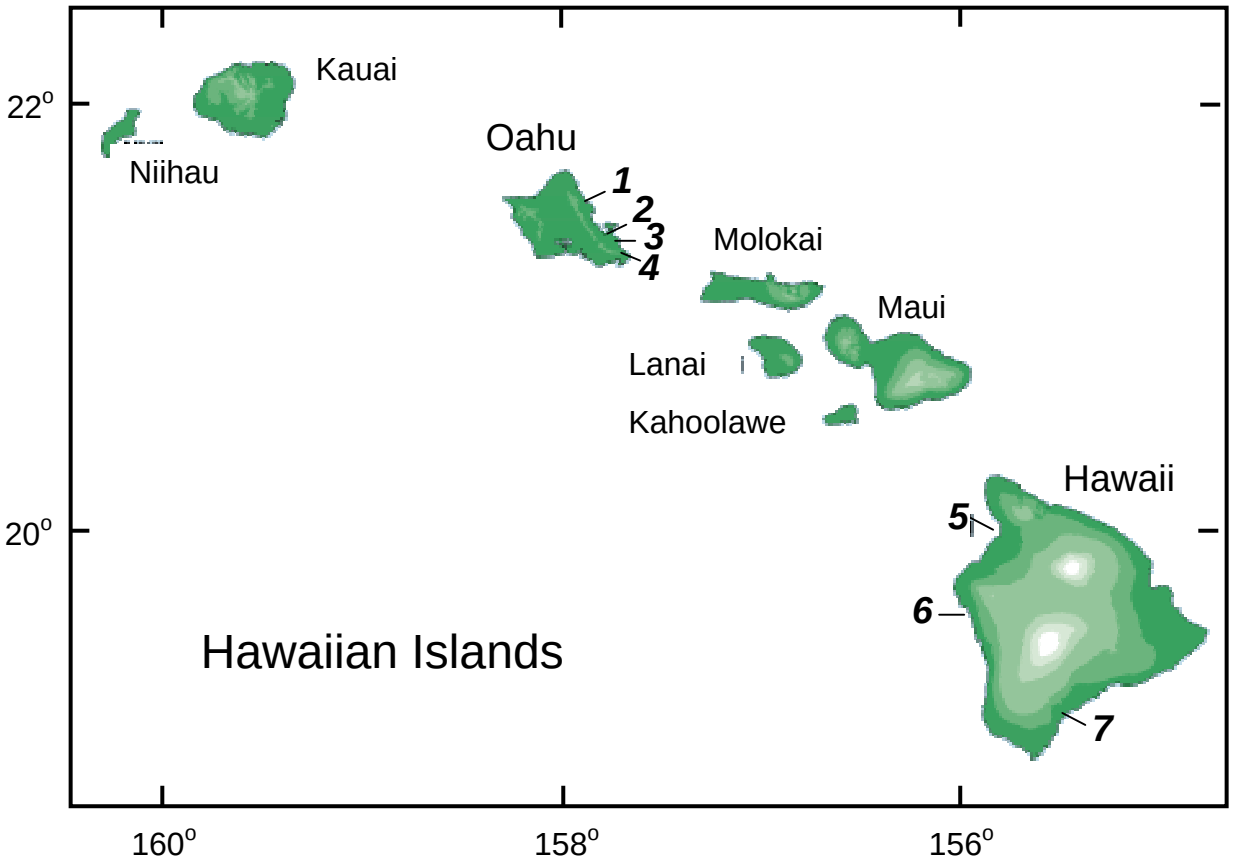

Figure 1. Capture location of Pacific threadfin specimens assayed in this study. For Oahu and Hawaii, regional sample locations and sample sizes are as follows: (1) Kaaawa, *n*=4; (2) Kailuana, *n*=17; (3) Kalama, *n*=5; (4) Waimanalo, *n*=6; (5) Hapuna, *n*=16; (6) Kialua, *n*=18; (7) Punaluu, *n*=7.

All PCR products were visualized on 1% TBE-agarose gels containing ethidium bromide (0.5 µg/mL). DNA bands were excised from the gels and purified using the QIAquick Gel Extraction Kit. Purified amplicons were ligated into vector DNA and used to transform competent JM109 cells which, in turn, were cultured on Luria-Bertani (LB)/ampicillin plates containing x-gal and IPTG. Blue/white selection was used to identify colonies potentially containing inserts. Plasmid DNA was isolated from minipreps using Wizard® *Plus* DNA Purification System (Promega, Inc.) and the presence of inserts was confirmed by digestion with EcoRI. Plasmid DNA was then purified using the PEG method described by Nicoletti and Condorelli (1993) and sequenced using a Licor 4200 automated sequencer. Sequence data were aligned with CLUSTALW (Higgins and Sharp, 1988).

Genetic Analyses

Levels of mtDNA control-region variability within samples of wild Pacific threadfin were examined by computing the nucleotide (Tamura and Nei, 1993) and haplotype (Nei, 1987)

diversity indices using the Arlequin 2.0b2 software package (Schneider *et al.*, 1999). Nei and Li's (1979) nucleotide divergence (*D*) values were estimated within and between groups. Statistical testing for population structuring involved 1) an *r* x *c* exact test (Raymond and Rousset, 1995) of a contingency table based on haplotype frequencies and 2) a hierarchical analysis of molecular variance (AMOVA; Excoffier *et al.*, 1992). Significance levels of all test statistics were evaluated by randomization testing as described in Arlequin. The level of migration (gene flow) between the islands was estimated from the fixation index (F_{ST}; Slatkin, 1991). Genealogical relationships between mtDNA control region haplotypes were reconstructed as follows: MEGA (Version 2.1, Kumar *et al.*, 2001) was used to generate pairwise matrices of corrected sequence divergence values (Tamura and Nei, 1993); the pairwise-deletion option for indels was invoked. From these, a neighbor-joining tree (Saitou and Nei, 1987) was constructed; statistical support for each node was obtained using the bootstrap procedure in MEGA.

To estimate female effective population size ($N_{e,f}$) for wild Pacific threadfin in Hawaii, the Metropolis-Hastings sampling method of Kuhner *et al.*, (1995), as implemented in FLUCTUATE (Version 1.3, mkkuhner@genetics.washington.edu), was applied to the entire set of mtDNA control-region sequences for wild specimens. Fu's (1997) F_S statistic and Tajima's (1989) *D* statistic were estimated using Arlequin. A mismatch distribution was constructed and analyzed for evidence of recent population expansion or bottleneck. Harpending's (1994) raggedness index (*r*) was computed for each distribution. Demographic parameters (and their associated SSD *P* values) were estimated using a non-linear least squares approach (Schneider and Excoffier, 1999).

To test the hypothesis that the condition of opercular deformity in cultured threadfin hatchlings was associated with maternal relatedness, all pairs of assayed individuals were classified as follows: both individuals were deformed/related maternally; both deformed/ unrelated maternally; both normal/ related maternally; both normal/ unrelated maternally. The proportion of deformed individuals that shared a dam and the proportion of normal individuals that shared a dam were each compared to the null proportion (50%) using *V* tests (DeSalle *et al.*, 1987).

Results and Discussion

Intraspecific Diversity Levels in Wild Pacific Threadfin

Our data indicate that the mitochondrial DNA control region represents a good source of intraspecific genetic characters for threadfin studies. For the preliminary survey, 1,045 base pairs within the control region were routinely and consistently scored. From these, 105 sites were polymorphic, including 3 sites that contained indels. The transition/transversion ratio was 4:1. A 10-base-pair sliding window (Fig. 2) shows the distribution of nucleotide substitutions over the DNA region assayed. Although substitutions occurred over the entire length of the sequenced segment, a conserved block exists in the center of the segment from which internal (nested) PCR primers could be developed if needed. There were no hypervariable areas within the threadfin control region. This represents a fortunate circumstance from the standpoint that mutational "hotspots" may confound the analytical models employed herein due to backmutations, among-site rate heterogeneity, and other violations of model assumptions. Additionally, it suggests that the control region should be a source of useful phylogenetic/taxonomic characters for morphologically similar *Polydactylus* (*see* Introduction).

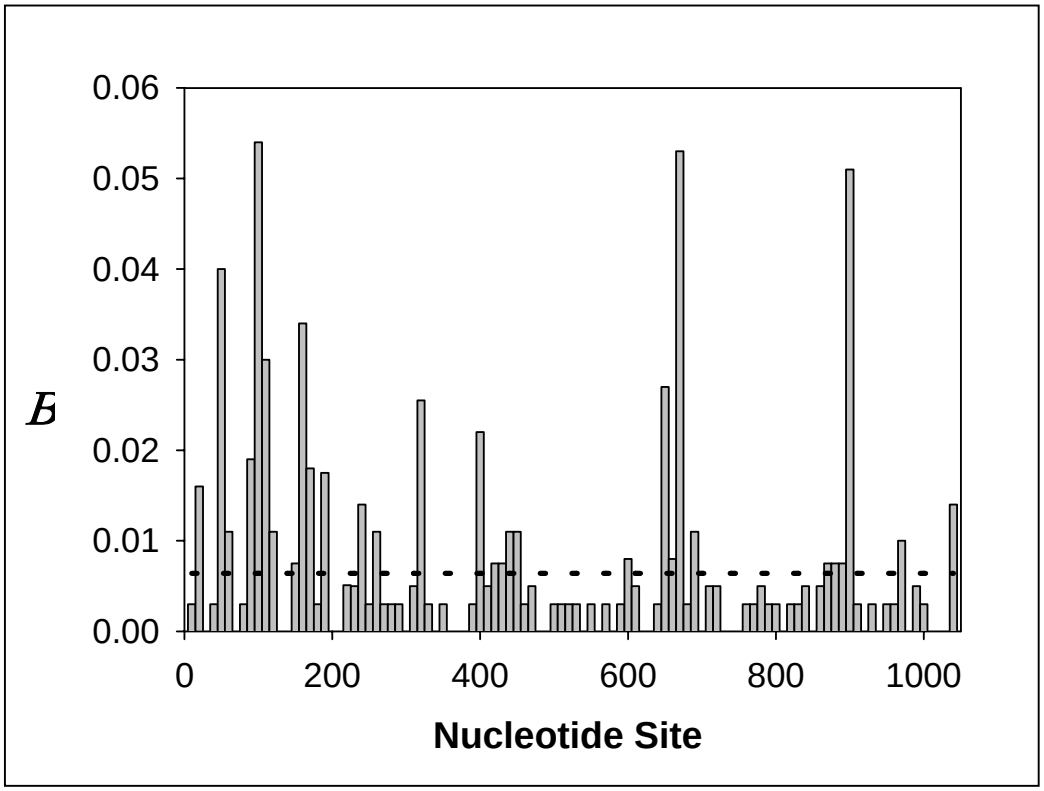

Figure 2. Ten base-pair sliding window of mitochondrial DNA control region variability for all wild Pacific threadfin. The dashed line depicts the mean value of nucleotide diversity (B=0.0064).

Among the wild specimens, 61 unique haplotypes were observed. Haplotype diversity (the probability of randomly choosing two individuals bearing different haplotypes) was high (h=99.3%). However, for wild Pacific threadfin, nucleotide diversity (the average number of pairwise differences among haplotypes per site) was somewhat low (π=0.0064), especially in comparison to that commonly observed in control regions of other marine percoids (e.g., *Sciaenops ocellatus* – 0.030 [Seyoum *et al.*, 2000]; *Lutjanus campechanus* – 0.025 [Garber, 2001]; *Cynoscion nebulosus* – 0.024 and *Archosargus probatocephalus* – 0.039 [S. Seyoum, M. Tringali and T. Bert, unpublished data]). However, the value was in keeping with levels of control region nucleotide diversity reported for three of four species of Hawaiian amphidromous fishes (range 0.004 to 0.010; Chubb *et al.*, 1998). The greatest genetic distance between any one pair of Pacific threadfin haplotypes was 0.018. Potential reasons for the low levels of "per site" mtDNA variation in threadfin are discussed below. Despite these low levels, sufficient marker variation existed for analyses of genetic structure, gene flow, and historical demography because of the large number of polymorphic sites assayed.

Genetic Structure and Gene Flow

The first step in effective fisheries management is to identify the "unit stock." The dynamics of organisms within this management unit will be driven by the same natural and

anthropogenic processes and respond similarly to regulation; however, the response of organisms in one unit stock will be independent of (although not necessarily different from) the response in another. Similarly, the first step in a program of genetic management is to identify the "genetic" stock(s) – i.e., the discrete gene pool(s) into which cultured organisms will reproductively integrate. Geographic delineation of genetic stocks structure in Hawaiian Pacific threadfin will permit informed management of Type I genetic impacts and establish hierarchical levels at which Type III impacts should be considered.

For statistical analysis of threadfin genetic structure, samples collected from locations within the islands of Hawaii and Oahu, respectively, were pooled *a priori*; the "Hawaii" group (n=41) was compared to the "Oahu" group (n=32). Sizes of samples within these groups are as yet too small to make valid intra-group comparisons. Average numbers of pairwise nucleotide differences for the Hawaii and Oahu groups were 7.646 and 5.312, respectively. The average pairwise difference between these two groups was 6.590. Because the between-group value was approximately the same as within-group values, a hypothesis of divergence between the groups was unsupported. Likewise, the result of the exact test for the geographic distribution of haplotypes was consistent with a hypothesis of panmixia (P=1.000 ± 0.000). The majority of individuals (n=54) had unique haplotypes. Thirty-four unique haplotypes were present among individuals in the Hawaii group; 20 were present among individuals in the Oahu group. The distribution of the 7 haplotypes that were shared within and between groups is given in Table 1.

Table 1. Distribution of shared mtDNA control region haplotypes within and between samples of Pacific threadfin, *Polydactylus sexfilis*, from the Hawaii and Oahu groups. See Fig. 1 for the composition of samples for the two groups. Fifty-four haplotypes were present in single individuals and are not listed here.

Haplotype Designation	Hawaii Group	Oahu Group
PT-006	2	2
PT-017	2	0
PT-022	1	1
PT-030	1	1
PT-039	1	2
PT-042	0	2
PT-045	0	4

There was no evidence of between-group geographic isolation in the population genealogy reconstructed using the neighbor-joining algorithm (Fig. 3). The genealogy was essentially star-like; shallow lineages were predominant. All bootstrap values on tree nodes (not shown) were less than 95% and typically less than 50%. The tree topology was geographically diffuse; neighboring haplotypes were often collected from different islands.

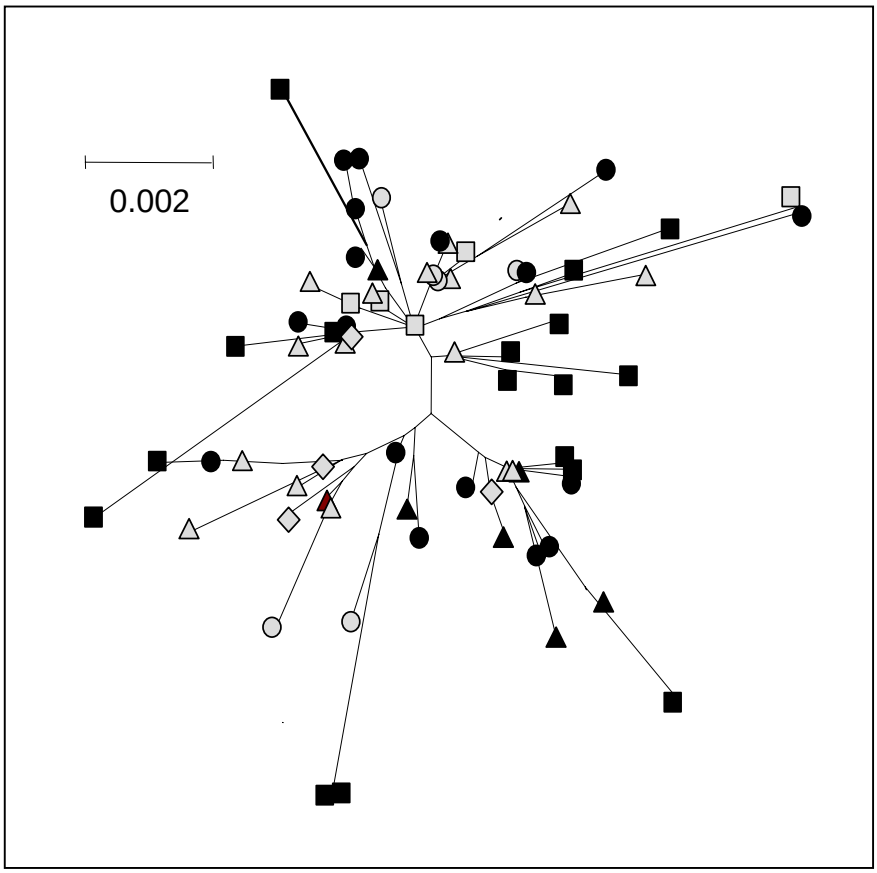

Figure 3. Neighbor-joining cluster of mitochondrial DNA control region haplotypes for all wild Pacific threadfin based on Tamura-Nei (1993) pairwise distances. Geographic locations from which haplotypes were obtained are depicted as follows: Oahu; Kalama, *grey squares*, Waimanalo, *grey circles*, Kailuana, *grey triangles*, Kaaawa, *grey diamonds*. Hawaii, , Kialua, *black squares,* Hapuna black circles, *Punaluu,* black triangles.

AMOVA was used to partition molecular variance within and between the Hawaii and Oahu groups (Table 2). Approximately 1.5% of the overall variance was due to differences between the two groups. Statistical significance of the observed between-group variance was tested by permuting haplotypes among groups; there was a 6.5% (i.e., reasonable) probability of obtaining a greater or equivalent between-group variance by chance alone. Ultimately, larger sample sizes for locations within islands will permit computation of within-island variance components for Hawaii and Oahu threadfin, respectively. If the hypothesis of panmixia is valid, these variance components would also be expected to be on order of 1-2%. The fixation index was low (F_{ST} = 0.0153, P = 0.108), suggesting that gene flow is high between these two groups and thus high for Pacific threadfin on a regional basis.

Table 2. Analysis of molecular variance (AMOVA, Excoffier *et al.*, 1992) for mitochondrial DNA control region haplotypes of Pacific threadfin from Hawaii.

Source of Variation	df	Sum of Squares	Variance Component	Percentage of Variation
Among Groups	1	5.165	0.05152	1.53*
Within Groups	71	235.266	3.31361	98.47

* *The probability that a larger value would have occurred by chance was 0.06549.*

To investigate the effects that reducing the length of the assayed marker would have on analytical results, the above analyses were repeated using only the first 500 nucleotides. In this case, the average numbers of pairwise nucleotide differences within the Hawaii and Oahu groups were 4.534 and 3.168, respectively and the average pairwise difference between these two groups was 3.908. Compared to the above divergence values, these values were reduced approximately by a factor of 2, as expected due to the reduction in the number of sites examined. But again, a hypothesis of divergence between the two groups was not supported. The test result for the geographic distribution of haplotypes was again consistent with a hypothesis of panmixia (P=1.000 ±0.000). In the AMOVA, 1.3% of the overall variance was due to differences between the two groups. There was a slight loss of statistical power in this AMOVA; the probability of obtaining a greater or equivalent between-group variance component by chance alone was 9.7%. The fixation index remained low (F_{ST} = 0.0129, P=0.090). In general, these results show that the length of the marker could be reduced as described without significant loss of resolution for extended studies of gene flow and genetic structure in threadfin. Additional resolution would more likely be obtained by the inclusion of more specimens in each of the existing samples. The shortened segment would be easily obtainable using *ThreadPro* and an internal primer. Shortening the length of the marker via an internal primer would reduce laboratory effort and expense since sequence could be obtained directly from amplicons. The model used herein to estimate female effective population size (*see below*) is sensitive to the number of characters (nucleotides) employed and the analysis benefited from the large size of the segment.

Overall, the preliminary results indicate that the mitochondrial genomes of Pacific threadfin from the islands of Hawaii and Oahu are not distinct. This circumstance can arise from two scenarios. First, it is possible that female reproductive exchange has occurred with sufficient frequency over time to homogenize or prevent divergence in threadfin mitochondrial genomes within the sampled range. If so, then the genetic sampling in this study has occurred within the geographic boundaries of a single genetic stock. Alternatively, it is possible that, if Oahu and Hawaii threadfin are indeed reproductively isolated from one another, that insufficient evolutionary time has passed since separation for significant genomic-level mtDNA divergence. Larger sample sizes obtained from the current island locations will be helpful in discriminating between these scenarios. For now, absent evidence of genetic stock structure in Hawaiian Pacific

threadfin, all wild specimens were pooled for analyses of female effective population size and demographic history.

Female Effective Population Size

The "effective population size" (N_e) is an important parameter for population genetic surveys (see Tringali and Bert, 1998). This parameter is related to the rate at which genetic change (e.g., inbreeding, drift, allelle fixation or loss) is expected to occur in a population. In this study, the mtDNA sequences from the 73 wild individuals were used to obtain an estimate of the female effective size ($N_{e,f}$) of the sampled Pacific threadfin genetic population. The method described in Kuhner *et al.* (1995) was employed. Briefly, the distribution of time intervals between coalescent events in mtDNA geneologies inferred from a population sample is shaped by the parameter 1. This parameter is related to the female effective population size and the mutation rate (*:*) of the sequenced DNA segment (i.e, for a haploid gene, $1 = 2\ N_{e,f}\ :$). A Markov-chain sampling of the genealogies was used to compute the maximum likelihood estimate of 1 = 0.655, which applies to the time of population sampling. The corresponding estimate of $N_{e,f}$ is relatively insensitive to the mutation rate (per site) of the sequenced marker. Adopting a very low divergence rate of 2% per million years, *:* equates to 8.5×10^{-7} (assumes a generation time of 6 years for threadfin). Adopting the highest recorded divergence rate for the control region in vertebrates (32% per million years; Merilä *et al.* 1997), $: = 1.6 \times 10^{-6}$. Thus, it is likely that the female effective size of the Pacific threadfin population at present is between approximately 205,000 and 385,000 individuals. We note that the range of values given here does not represent a confidence interval; rather, it reflects uncertainty in the mutation rate for threadfin. The approach of Kuhner *et al.* (1995) accounts for changing population sizes. The maximum likelihood estimate of the population growth rate parameter, *g*, was 933.367 for threadfin. Positive values of *g* are indicative of net population growth (expansion) over time.

A large female effective size for Pacific threadfin in Hawaii would be a positive sign for the present genetic health of the population. Presumably the overall effective size (for both sexes) is correspondingly high. Maintenance of population fitness hinges on a population's ability to remove maladapted or lethal genes through the process of natural selection. Once introduced, the probability that a particular deleterious gene will persist or become fixed in a population is principally a function of its particular selective effect, the initial frequency at which it enters the population, and effective population sizes during the course of its segregation. In general, unless swamped by a multitude of related individuals having maladapted genomes, populations having large N_es will incorporate the majority of selectively beneficial genes and remove the majority of those that are deleterious. In contrast, populations having small N_es, unable to overcome the stochastic effects of drift, may suffer reduced levels of population fitness from an increased mutation load and/or disruption of coadapted genomes. The expectation from the above results is that the present mutation load in Hawaiian Pacific threadfin is low.

Demographic History of Pacific Threadfin in the Hawaiian Islands

Over the long-term, the loss of adaptive variation and the accumulation of deleterious mutations represent potential hazards to population viability. Significant risk from these hazards may exist in populations having undergone significant periods of low N_e, even if current N_es are high. The evolutionary age of the population is also a factor – older populations are expected to have a larger mutation load than younger populations of similar effective size due to the accumulation of slightly deleterious alleles. Sudden occurrences of extreme inbreeding, as may

result from Types II and/or III stocking impacts, may be more problematic for populations having a large mutation load. Therefore, the demographic histories of populations significantly influence their long- and short-term susceptibilities to naturally or artificially induced genetic change. Fortunately, past demographic fluctuations (i.e., population expansions and contractions) often leave genetic signatures encoded in DNA sequence (reviewed in Harpending *et al.*, 1998).

For threadfin, the estimate of Fu's (1997) statistic was a very large, negative value (F_S = -73.295), which may indicate that there was a recent expansion in the sampled population. Figure 4 depicts values of Tajima's (1989) *D* over the assayed region of mtDNA. The overall estimate of Tajima's statistic (*D* = -2.33, *P* = 0.00193) was significantly different than the value expected under the neutral mutation hypothesis. This finding is consistent with hypotheses of marker non-neutrality or a recent founding/bottleneck event. Results from other studies of control region variation in (drift/mutation) equilibrium populations, including marine percoid shorefishes (e.g., *Sciaenops ocellatus*, *Cynoscion nebulosus*, and *Archosargus probatocephalus*; S. Seyoum and M. Tringali, unpublished data) are consistent with neutral mutation. Thus, unless there was a recent "sweep" of control-region variation (see Ballard and Kreitman, 1995) via hitchhiking, demographic explanations most likely apply to threadfin.

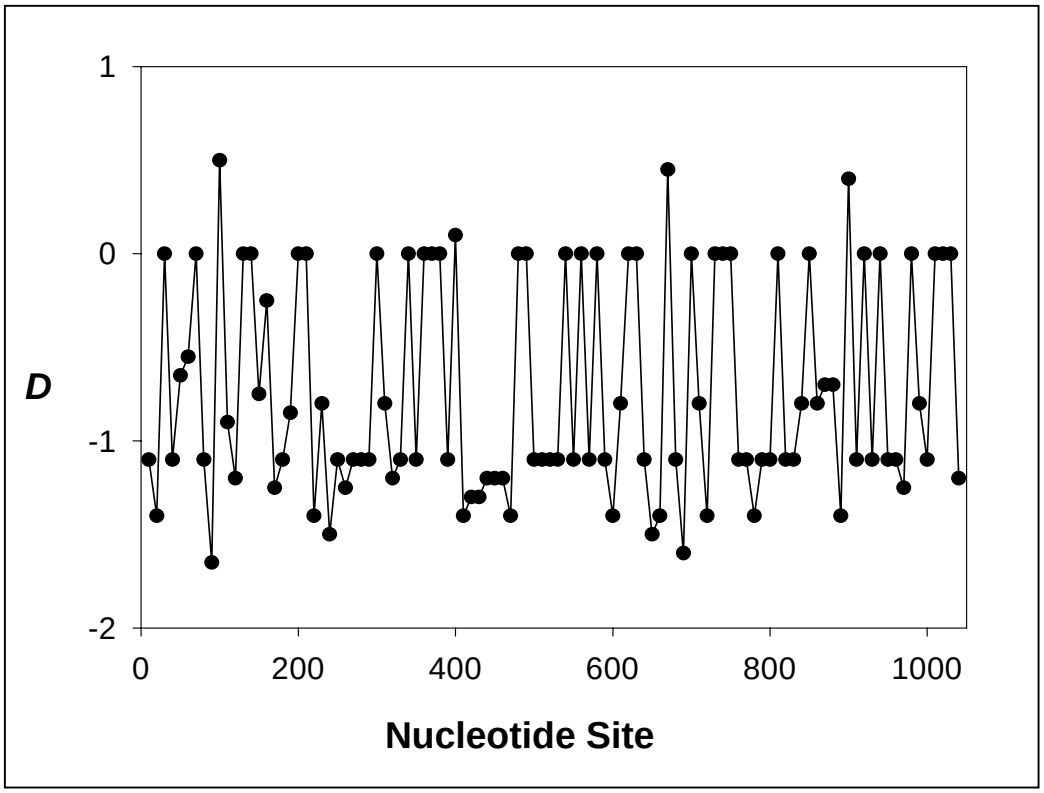

Figure 4. Ten base-pair sliding window showing values of Tajima's (1989) *D* for the assayed region of mtDNA control region in wild Pacific threadfin.

Figure 5 shows the observed and expected distributions of pairwise sequence differences (*i*) among threadfin specimens. The mean and variance of the observed distribution was 6.612 and 9.836, respectively. The observed data exhibited a good fit to that predicted by the "sudden expansion" model of Rogers (1995). The sum of square deviations between the observed and expected distributions (*SSD* = 0.0008) was not significantly different (*P* = 0.66) from a simulated distribution that assumes that the estimated parameters ϑ, τ_0, and τ_1 are the true ones (*see* Schneider and Excoffier, 1999). The mean square error (MSE) measuring the fit of the data to the sudden expansion model was 0.001131 whereas the MSE measuring its fit to an "equilibrium" (no growth) model was 0.050125 (see Merilä *et al.*, 1997). Harpending's raggedness index was very low (*r* = 0.007), indicating that the observed distribution was unimodal. Thus, as in the analyses of *g*, F_S, and *D*, the analysis of the mismatch distribution was consistent with a hypothesis that the sampled Pacific threadfin population recently underwent an expansion.

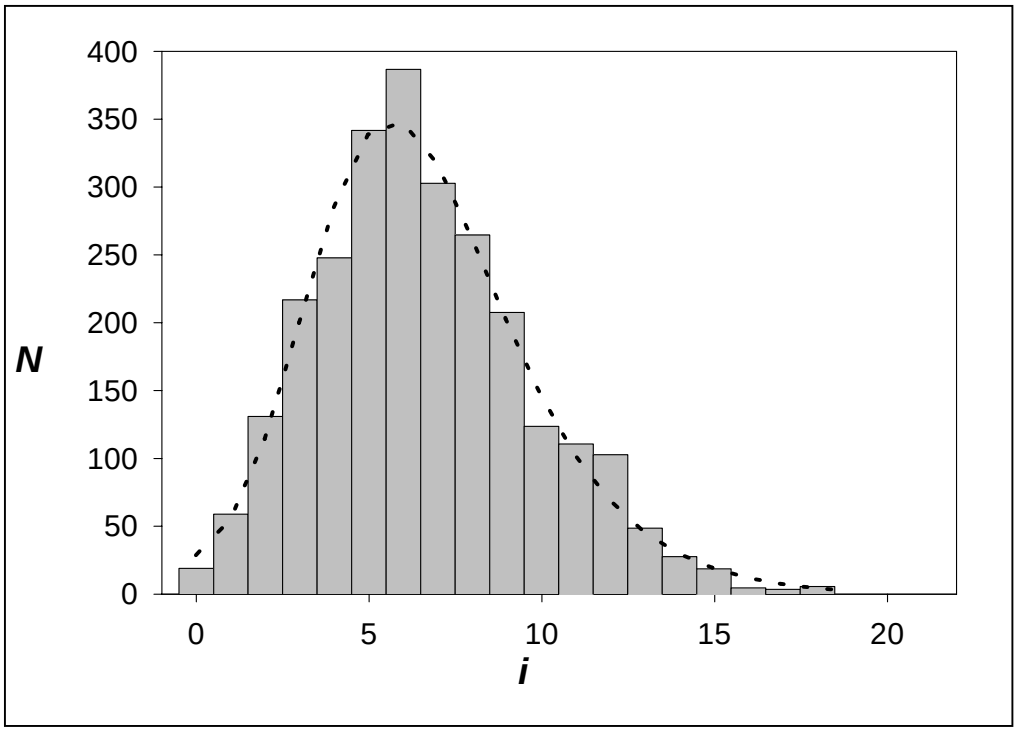

Figure 5. Distribution of the pairwise number of nucleotide differences among *N* pairs of individuals for wild Pacific threadfin. Empirical distribution represented by vertical bars. The expected distribution as predicted by the "sudden expansion" model of Rogers (1995) is depicted by the dashed line.

The demographic parameter ϑ represents a mutational time scale such that the number of generations that have elapsed since a population was founded is approximately ϑ divided by two times the overall (not per site) mutation rate (Rogers and Harpending, 1992). For the sampled Pacific threadfin population, ϑ was estimated to be 5.004. Applying the broad range of control-region divergence rates described in the above section, the estimated time of founding for the

sampled population was approximately 14,000 to 28,000 years ago. This range of founding times is interestingly low considering the presumptive geological ages of the Hawaiian Islands (from approximately 1 to 5 million years old for the southernmost to northernmost islands) and could be explained by at least three scenarios. First, Pacific threadfin may be a nascent species, in which case, the genealogy and diversity-level observed herein would be representative of samples assayed from throughout the entire Indo-Pacific range. This scenario would be testable via the addition of specimens from a distant location (e.g. Japan, Marshall Islands, Caroline Islands). Second, the sampled Hawaiian population (or, less likely, the entire species) could have undergone a severe bottleneck or selective sweep just prior to the estimated founding time. The estimates of τ_0 and τ_1 suggest that the bottleneck size would have been less than approximately 1000 females and the subsequent expansion size approximately two orders of magnitude greater than 1000 (which is consistent with the above estimate of $N_{e,f}$). However, the leading edge of the mismatch distribution is not consistent with a bottleneck scenario (Rogers and Harpending, 1992). That is, the leading edge of distributions in bottlenecked populations based on 1000 control region nucleotides would characteristically be expected to be ragged and, because of stochastic retention of older lineages, contain many peaks at values of $i > 25$. In threadfin, there are no peaks beyond $i = 18$ and the distribution is smooth. Third, the Hawaiian population could have been colonized (founded) recently by migrants from another Indo-Pacific population. This scenario is not unreasonable given Hawaii's well-known biogeographic history of fish fauna. It is also testable via comparison to a distant sample.

Etiology of Opercular Deformity in Pacific Threadfin

During captive propagation, OI threadfin hatchlings occasionally exhibit a developmental defect resulting in a partially or fully missing gill plate. Osteological and morpho-anatomical defects in cultured fishes can be caused by various factors, both genetic and nongenetic. Examples of commonly reported nongenetic factors include water quality, nutrition, water temperature (particularly during larval rearing), and phenotypic maternal effects. Known genetic causes or factors include inbreeding depression, autosomal single locus variants, and various types of genotypic maternal effects. Often, multiple, interacting causes exist for a given condition. Opercular complex deformity (OCD) manifests in a variety of freshwater and marine cultured species, including cyprinids (Mrakovic and Haley, 1979), cichlids (Winemiller and Taylor, 1982; Soliman *et al.*, 1986; Wimberger, 1993; Tave and Handwerker, 1994), salmonoids (Jensen, 1988; Harris and Hulsman, 1991), ictalurids (Lim and Lovell, 1978), and sparids (Koumoundouros *et al.*, 1997). It is also known in wild fishes (Valentine and Bridges, 1969; Lindesjöö *et al.*, 1994). Although the condition has been etiologically related to nutritional factors, particularly dietary ascorbic acid (vitamin C), other causes or contributing factors have been posited (e.g., pollutants, behavior, water current, water gas supersaturation). Not surprisingly, affected individuals exhibit poor growth/survival and increased disease susceptibility.

Mair (1992) found that opercular deformities were sometimes associated with a lethal condition known as caudal deformity syndrome (CDS) in *Tilapia* (=*Oreochromis*) *niloticus*. CDS is known to be under the control of an autosomal recessive gene, thus, more likely to occur in inbred tilapia. However, Tave and Handwerker (1994) demonstrated by breeding studies that OCD, when not associated with CDS, was non-heritable in *O. niloticus*. OCD presents after 4-5 generations of inbreeding in *Cichlasoma nigrofasciatum* (Winemiller and Taylor, 1982) and *Brachydanio rerio* (Mrakovic and Haley, 1979), suggesting that there is an underlying genetic cause in these two fishes. In captive breeding at OI, the condition appears in F1 progeny of

parental broodstock that are obtained directly from the wild population. If an underlying genetic factor is responsible for OCD in cultured threadin, then stocking progeny groups that contain affected hatchlings represents a potential Type II genetic hazard. Here, we report the result of a genetic assay in which the relationship between opercular deformity and maternal relatedness was investigated.

Two OI spawning groups, each composed of approximately 15 sires and dams, respectively, produced fertilized eggs in mass spawning events. Zygotes from these two spawning groups were mixed in approximately equal proportions, divided equally into four tanks, and reared for 25 days. Hatchlings from all four tanks were pooled into two nursery tanks and pooled again into one group prior to size gradation. From this mixed hatchling group, 24 specimens having deformed opercles and 25 developmentally normal specimens were collected for DNA analysis. Because OI broodstock are obtained from wild stock, inbreeding coefficients (levels of relatedness) among breeding pairs and among pairs of dams were assumed to be low. Haplotypes of potentially contributing females are as yet unknown; maternity was inferred from mtDNA control region haplotypes of hatchlings. The haplotype diversity level observed in the survey of wild threadfin suggests that the preponderance of OI female broodstock, which are collected from the wild, will have unique haplotypes. Taq error, inherent in the cloning step of DNA sequencing, may have caused a few maternally related individuals to be scored as unrelated; however, this phenomenon would be expected to occur randomly between deformed and normal hatchlings.

If there were no Taq errors, then at least 21 different dams contributed to the overall hatchling group, although contributions from other females may have been missed due to binomial sampling error. Relative maternal contributions to the sample of 49 hatchlings varied from 1 to 22 offspring per dam. Of the 22 hatchlings that shared one of the dams, 11 were deformed and 11 were normal. In 2 of the 3 other cases for which multiple individuals shared a particular dam, both normal and deformed hatchlings were observed. Results for the pairwise comparisons of cultured individuals (*described in* Material and Methods) are as follows: both deformed/related maternally – 56; both deformed/ unrelated maternally – 244; both normal/ related maternally – 84; both normal/ unrelated maternally – 192. If the deformity were positively associated with maternal relatedness, then we would expect the proportion of deformed individuals that shared a dam to be greater than the proportion of normal individuals that shared a dam. If the condition occurred randomly with respect to maternal relatedness, then the expected (null) proportion for both groups would be 50%. There appeared to be no positive association between the deformity and maternity. That is, among pairs of individuals that shared a dam, both individuals were deformed in 40% (56/140) of the comparisons and both were normal in 60% (84/140) of the comparisons. There was not a statistical difference between the above proportions and the expectation (V=2.79; $P > 0.1$ for both tests). Thus, these preliminary results offer no evidence that inbreeding and maternal effects were causes for the defect. However, the results could have been confounded if some of the contributing dams shared haplotypes.

We cannot fully dismiss the possibility that OCD has an underlying genetic cause. However, our data shed light on the potential for genetic impact on wild threadfin from stocking if the most common genetic factor – autosomal monogenic inheritance – has a role. *Penetrance* refers to the proportion of individuals that show an expected phenotype under a specific set of environmental conditions. *Expressivity* refers to the range of phenotypes expressed under a set of environmental conditions or over a range of environmental conditions. If genetically controlled, it is possible that a trait such as OCD will exhibit variable penetrance and/or expressivity – e.g., low penetrance/expressivity under wild conditions and high penetrance/expressivity during

captive breeding/rearing. Table 3 outlines phenotypic expectations for a hypothetical OCD variant for several forms of autosomal monogenic inheritance modes over a range of environmentally controlled penetrance values. We may generate several inferences using this table, our maternity data, and field observations of wild threadfin. OCD has not been observed in wild threadfin populations; thus, the morphological condition was neither expected nor apparent in any OI broodstock. Yet progeny of at least 13 different dams (more than one half of putatively contributing dams) were affected by OCD. If the trait were under the control of a recessive allele, then a very large proportion (approximately half) of the contributing dams (and one or more sire) would have had to have been heterozygous or phenotypically normal homozygous carriers of that allele. This would undoubtedly require that the allele be common in the wild population from which the broodstock were collected. If the allele were common in wild threadfin, the phenotypic expression of it would be not uncommon unless natural penetrance/expressivity was extremely low. Similarly, the various hypothetical dominance modes of gene action would also require naturally low penetrance/expressivity because the condition presents itself recurrently at OI in F1 progeny of phenotypically normal broodstock obtained directly from the wild population. More complex genetic mechanisms, e.g., certain sex-linked dynamics, epistatic interactions, and polygenic inheritance, cannot be ruled out without controlled breeding experiments.

Table 3. Phenotypic expectations for various modes of single-locus gene action for a hypothetical gene (*o*) that controls expression of opercular complex deformity. Wild-type alleles are designated by (+). The letter "p" represents the proportion of individuals of the specified genotype that exhibit the expected phenotype for a given set of environmental conditions. "Variable p" indicates that some form of expression occurs but varies in frequency of occurrence according to p.

Genotype	Penetrance of *o*		
	p = 100%	**100% > p > 0%**	**p = 0%**
A. Recessive *o*			
+ +	normal	normal	normal
+ *o*	normal	normal	normal
o o	deformed	deformed-variable p	normal
B. Dominant *o*			
+ +	normal	normal	normal
+ *o*	deformed	deformed-variable p	normal
o o	deformed	deformed-variable p	normal
C. Incompletely Dominant *o* (including additive)			
+ +	normal	normal	normal
+ *o*	partially deformed	partially deformed-variable p	normal
o o	deformed	deformed-variable p	normal
D. Incompletely Dominant Lethal *o*			
+ +	normal	normal	normal
+ *o*	deformed	deformed-variable p	normal
o o	dead	dead/deformed-variable p	normal

Selection acts on phenotypes. Thus, if OCD is under some form of simple autosomal genetic control for which there is low natural penetrance/expressivity, then the selection coefficient for the presumptive OCD gene in wild threadfin is expected to be very low unless the natural environment changes to a state that favors expression. From the standpoint of stock enhancement, this represents a low-risk scenario because the potential genetic impact, if any, would be purifying effect – captive breeding would expose individuals with OCD genotypes to selection. Reductions in allelic diversity at tightly linked gene loci would accompany this effect only if the wild population was swamped by stocked individuals. From the standpoint of other forms of threadfin aquaculture that may develop (e.g., commercial pond, cage, and net-pen culture), the above scenario, to the extent that the condition affects production and profitability, is somewhat more problematic. Mitigating the impact in this case would require either broodstock domestication or identification and elimination of exogenous factors that foster OCD expression.

To improve upon our initial experiment and eliminate sources of bias that may have been present, it is recommended that the experiment be replicated with samples from 2-3 additional progeny groups and include individual classification of degree of deformity in progeny. Left and right opercles of contributing broodstock should be retained for phenotypic evaluation as these individuals are cycled out of production. Markers that permit determination of both male and female parentage (e.g., 3-4 microsatellite loci) should be utilized if possible. Broodstock should be genotyped using finclips for DNA. If the condition remains randomly distributed among related and unrelated OI hatchlings within progeny groups and ubiquitous with respect to parental involvement, then critical data pertaining to reproductive variance and effective sizes of OI cohorts would be obtained simultaneously. Data pertaining to family size variance in OI progeny groups are needed to address Type III genetic concerns.

Concluding Remarks

From the standpoint of genetic management, the most significant finding in this preliminary genetic survey was that Pacific threadfin along (at least) the majority of the Hawaiian Island chain appear to comprise a single genetic stock. Maintenance of a larger gene pool via reproductive exchange between island populations may afford Pacific threadfin a degree of protection against the effects of drift and localized demographic stochasticity. Should the study of additional wild specimens support this finding, then the Type I genetic hazards identified by Tringali and Leber (1999) can be safely mitigated through the use of Hawaiian broodstock for threadfin stock enhancement, provided that impacts from Type II and III hazards are managed. Moreover, these Type I considerations can be extended to other forms of threadfin aquaculture that may occur in Hawaii, e.g., commercial pond, cage, and net-pen culture, particularly as they pertain to the issue of escapement. It will be important to ultimately resolve the evolutionary size and age of the Hawaiian threadfin population via outgroup sampling. If it turns out that threadfin colonized the island chain approximately 10^3 generations ago and maintained historically large effective sizes during that period, then the present population should not be overly burdened by segregating deleterious mutations (Lande, 1994), although that would require confirmation *via* crossbreeding studies. Nonetheless, given the current low abundances at some locations within the Hawaiian threadfin population and an overall low level of genetic variation putatively associated with a recent founding, maintenance of natural allelic

composition and adaptive diversity during large scale stocking efforts will require attention to broodstock numbers (N_e of cohorts) and post-stocking hatchery/wild ratios.

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